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Micro-Iodometric Assay for Penicillinase

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Penicillinase activity can be reliably estimated iodometrically (Perret, 1954; Ghosh & Borkar, 1961), manometrically (Henry & Housewright, 1947), and alkalimetrically (Wise & Twigg, 1950), as well as by bio-assay (Pollock, 1956). However, staphylococcal penicillinase has so great an affinity for its substrate (Novick, 1962) that only the bio-assay is sensitive at the low substrate concentrations necessary for the study of enzyme kinetics. The bio-assay has been used successfully for the estimation of the Michaelis constant (K_m) of *Bacillus cereus* penicillinase (Pollock, 1956), but it is unwieldly and time-consuming and is unsuitable for the precise measurement of initial reaction velocities because, to be reliable, any single measurement must be based on the destruction of at least one-quarter of the substrate initially present.

The assay procedure described was developed for the purpose of measuring the rate of hydrolysis of penicillin to penicilloic acid by penicillinase at substrate concentrations as low as μM. The method depends, like other iodometric procedures, on the reduction of iodine by penicilloic acid but not by penicillin. Reaction velocities were estimated by measuring the rate of decolorization of the starch–iodide complex by penicilloic acid when the reaction took place in a medium containing starch and iodine; by this means, a 1000-fold increase in sensitivity over existing techniques was obtained.

MATERIALS AND METHODS

Reagents

Penicillinase, prepared from *Staphylococcus aureus* 524SC (Novick, 1962), was used in these experiments. It has maximum enzymic activity at pH 5·8 (Novick, 1962). Enzyme preparations should have less than 20 mg. of protein/unit of penicillinase activity; 1 unit catalyses the hydrolysis of 1 μmole of penicillin G in 1 hr. at 30° and at pH 5·8.

Phosphate buffer (0·05M), pH 5·8, contained 6·25 g. of KH_2PO_4 and 0·696 g. of K_2HPO_4/l.

Penicillin G (sodium salt), from Glaxo Laboratories Ltd., Greenford, Middlesex, 2·5 mM in phosphate buffer, was made up fresh daily and kept on ice.

Hydrolysed-starch soln., 2·0% (w/v) (Smithies, 1955) was made by suspending the powder from Connaught Medical Research Laboratories, University of Toronto, Toronto, Ontario, in the phosphate buffer at room temperature, heating to boiling over an open flame with constant swirling, boiling gently for 2–5 min. until clear, and then cooling to room temperature. Starch solution became cloudy on standing and was prepared fresh daily. The choice of starch is important because the iodine adsorption isotherm and the colour of the complex vary considerably according to starch structure. The most satisfactory material is amylose of mol.wt. over 10 000. Its advantage over starches which are highly branched and heterogeneous with respect to chain length is that it gives a pure-blue iodide (Swanson, 1948), solutions of which have an absorption maximum which does not change with iodine concentration (Baldwin, Bear & Rundle, 1944) and which have extinctions that are a linear function of iodine concentration. Also the adsorption isotherm of amylose and iodine is characterized by a constant low free-iodine activity over a wide range of total-iodine concentrations (Gilbert & Marriott, 1948). The starch preparation of Smithies has properties sufficiently close to those of amylose to give satisfactory results and was used because it is available in bulk.

Iodine, 0·08M in 3·2M-potassium iodide, was stored in a brown bottle. To make 200 ml. of starch–iodide solution, 0·30 ml. of iodine–potassium iodide was mixed with 180 ml. of the phosphate buffer, and 20 ml. of the 2% starch solution added slowly with stirring. The final iodine concentration was thus 120 μM. Colour was the most reproducible and full colour development was the most rapid, requiring about 15 min. at room temperature, when starch was added to previously diluted iodine. The colour was stable for at least 6 hr. at room temperature, but decreased slowly on prolonged standing.

Procedure

For a typical experiment, into each of two 1 cm. glass cuvettes were pipetted 1·0 ml. of starch–iodide, 1·0 ml. of 0·03–0·6 mM-penicillin G, 0·9 ml. of phosphate buffer, and 0·2 ml. of 2% starch soln. The cuvettes were then placed in a Unicam spectrophotometer (SP. 500) fitted with thermostat control and kept at 30°. After 5 min. the reaction was started by adding to one of the cuvettes 0·1 ml. of enzyme with 0·02–0·2 unit of activity. After 15 sec. the same amount of heat-inactivated enzyme was added to the other cuvette. (Heat inactivation was accomplished by autoclaving at 120° for 20 min.) The extinction was then recorded at 620 mμ every 30 sec. by reading the control cuvette against the reaction cuvette. This procedure gave readings that increased during the decolorization reaction.

For greater flexibility, concentrations of reactants could be varied. To give the greatest accuracy, they were chosen

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Table 1. *Lengths of light-path and convenient concentrations of reactants*

Light-path (cm.)	Concn. of iodine (μM)	Range of substrate concn. (μM)	Range of enzyme concn. (units/l.)
4·00	13	2·5–50	5–50
2·00	20	5–100	10–100
1·00	40	10–200	20–200
0·50	80	20–400	40–400
0·25	160	40–800	80–800

so that readings covered as much of the range of the instrument as possible. Thus an iodine concentration was chosen that gave an extinction of 1·2 against a water blank, and enough substrate used to give complete decolorization. When the substrate concentration was too great, spontaneous hydrolysis produced a high blank which decreased the accuracy. Therefore, for substrate concentrations outside the range given above, the length of the light-path was changed by using different-sized cuvettes and iodine concentration was altered concomitantly. Table 1 lists the different light-paths used with the initial iodine concentration which gave an extinction of 1·2 with each, and the range of enzyme concentrations that were found convenient. Each range of enzyme concentrations listed was chosen to give complete decolorization in 5–50 min. with saturating substrate. As the Unicam instrument was not supplied with different-sized cuvettes, for measurements with light-paths other than 1 cm. a Hilger Spekker absorptiometer was used with a yellow (Ilford no. 606) filter. As this instrument did not have thermostat control, reactions were run in large volumes in a water bath at 30°, samples being discarded after reading. Within the limits of experimental error, the same results were obtained with the Spekker as with the Unicam instrument.

RESULTS

Rates of decolorization of starch–iodide during penicillin hydrolysis were converted into enzyme reaction velocities by the formula

$$v = \frac{\Delta E}{lP\Delta t} \qquad (1)$$

where v is μmoles of substrate hydrolysed/l./hr., ΔE is change in extinction during time Δt in hr., l is length of light-path in cm., and P is the change in extinction over a 1 cm. light-path which results from the addition of 1 μmole of penicilloic acid to 1 l. of starch–iodide 40 μM with respect to iodine. P is related to ϵ_I, the molar extinction coefficient of iodine in starch–iodide, approximately by

$$\tfrac{1}{4} \times P \times 10^6 = \epsilon_I$$

because approximately 4 moles of iodine are reduced by 1 mole of penicilloic acid, and because ϵ_I refers to molar units and P to μmolar units.

Iodine extinction coefficient. The complex between iodine and the starch preparation described had an absorption maximum at 620 mμ. The wavelength of the absorption maximum was independent of iodine concentration, but there exists a threshold iodine concentration below which the complex was almost colourless (Lambert, 1951). Between the threshold concentration (3 μM-iodine) and a concentration 50-fold greater, increase in extinction was a linear function of the amount of iodine added. Therefore, although the complex did not obey Beer's Law, the molar extinction coefficient, ϵ_I, for iodine in starch–iodide at constant starch concentration could be calculated according to the equation (Lambert, 1951)

$$\epsilon_I = \frac{E}{(C - C_0)\, l} \qquad (2)$$

where E is the extinction at 620 mμ, C is the iodine concentration in moles/l., C_0 is the threshold concentration, and l is the length of the light-path in cm. The value obtained with the Unicam spectrophotometer was ϵ_I = 34 200 at 30°. Because the relationship between starch, iodine, and the blue colour of the complex is not fully understood, it could not be assumed that the observed proportionality of extinction to iodine concentration would hold during the removal of iodine from the complex. To test this, different amounts of standard sodium thiosulphate solution were added to equal portions of starch–iodide and extinctions were determined immediately. The resulting curve of extinction as a function of thiosulphate concentration could be compared with the iodine standard curve because 2 moles of thiosulphate are equivalent to 1 mole of iodine. The curves for the addition of iodine to the complex and its removal by thiosulphate were superimposable; therefore, the colour change produced by the addition of iodine to starch was precisely reversible.

Determination of P. As mentioned above, P is the product of the iodine extinction coefficient and the iodine equivalent of penicilloic acid. However, the stoicheiometry of the reaction between penicilloic acid and iodine has not been precisely established, since published values for the number of equivalents of iodine reduced by 1 mole of penicilloic acid differ by as much as 10% (Scudi & Woodruff, 1949; Perret, 1954). Therefore, P was determined under conditions which resembled as closely as possible those of the assay by allowing the enzymic hydrolysis of penicillin to proceed to completion in the presence of excess starch–iodide. A linear relationship between final extinction and initial penicillin concentration was obtained and the slope of the line gave a value for P of 0·128.

Dependence of velocity on enzyme concentration. When a series of reactions was run with different amounts of enzyme and a constant amount of substrate, the curves shown in Fig. 1 were obtained.

It should be noted that after a lag period of 3–4 min. decolorization rates were linear. Reaction velocities, v, calculated from the slopes of the linear portions of the curves according to equation 1, were found to be a linear function of enzyme concentration. However, when the activity of a given enzyme sample was measured by the micro-assay and by the standard assay of Perret (1954), the reaction velocity obtained with the micro-assay was about 40% lower than that obtained with the standard assay. The discrepancy could not be accounted for by the lower substrate concentration used in the micro-assay because at that substrate concentration (50 μM) the reaction velocity was 98% of the maximum velocity.

Effect of starch and iodine on the enzyme. The possibility that starch or iodine was responsible for the low enzyme activity was examined. For this purpose enzyme (final concn. 0·2 unit/ml.) was incubated with starch–iodide (iodine concn. 0·16 mM) at 30° in the absence of substrate and samples were removed periodically and assayed by the micro-assay. There was no detectable diminution in activity over a period of 3 hr. A further experiment was done in which two reaction flasks containing the same concentrations of substrate (50 μM), enzyme (0·05 unit/ml.), potassium iodide (3·2 mM) and starch (0·2%) in a total volume of 100 ml. were incubated in parallel. To one, iodine (final concn. 80 μM) was added 1 min. after the enzyme. To the other, the same amount of iodine was added 19 min. after the enzyme, when the decolorization in the first flask was approximately half complete. The results of this experiment (Fig. 2) show no detectable difference in activity between enzyme acting in the absence of iodine and enzyme acting in its presence. The broken line is a theoretical curve plotted on the assumption that the reaction velocity in the absence of iodine was 40% greater than in its presence. It is apparent that, if this had been so, it would have been readily detectable. The effect of starch on the activity of penicillinase was examined by incubating enzyme (2·0 units/ml.) with substrate (5 mM) in 10 ml. of 0·2M-phosphate buffer, pH 5·8, both with and without the addition of starch (final concn. 0·2%). The amount of penicilloic acid produced in 10 min. at 30° was determined by the standard iodometric assay of Perret (1954) and was the same with and without starch.

Thus the 40% lower penicillinase activities obtained with the micro-assay cannot be accounted for on the basis of any effect of starch or of iodine on the enzyme.

The micro-assay has been used for the measurement of K_m of staphylococcal penicillinase with penicillin G and with α-phenoxymethyl-, α-phen-

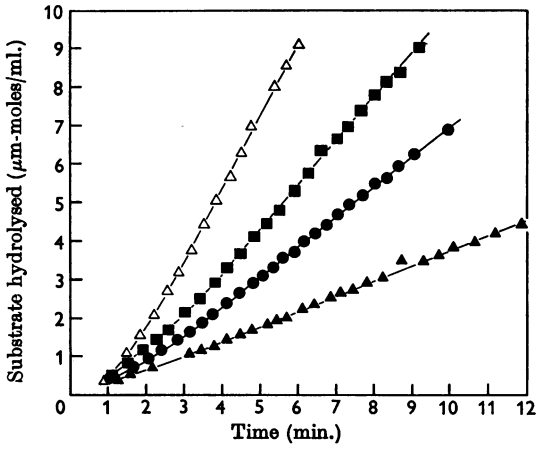

Fig. 1. Penicillin–penicillinase reaction rates. For each curve, 1·0 ml. of starch–iodide (0·2% starch, 120 μM-iodine, 1·6 mM-potassium iodide) was incubated with 1·0 ml. of 0·150 mM-penicillin G in a 1 cm. cuvette at 30° for 5 min. Enzyme (activity previously determined by standard assay) was then added to give final concentrations (units/ml.) of 0·04 (▲); 0·08 (●); 0·12 (■); and 0·18 (△). On the ordinate scale are plotted amounts of substrate hydrolysed/ml., obtained by dividing extinctions by P.

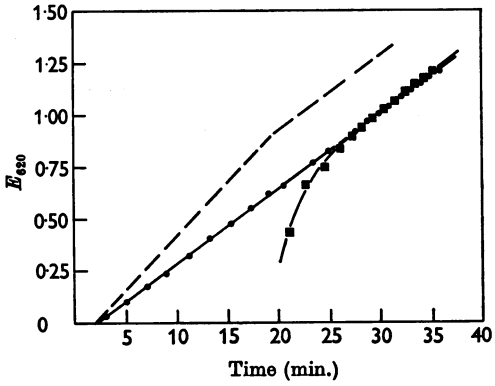

Fig. 2. Effect of iodine on staphylococcal penicillinase. Reaction volumes were 100 ml. each flask containing 5 μmoles of penicillin G, 5 units of penicillinase activity, 0·2 g. of starch, 320 μmoles of potassium iodide and 5 m-moles of potassium phosphate buffer, pH 5·8. Enzyme was added last. After 1 min., 8 μmoles of iodine were added to one of the flasks (●). To the other flask (■), the same amount of iodine was added 19 min. after the enzyme. Incubation was carried out at 30° and extinctions were recorded in a Spekker absorptiometer, with 5 mm. cuvettes. A theoretical curve (broken line) was plotted on the assumption that the reaction in the second flask (■) proceeded at a rate 40% greater than that in the first (●) until the iodine was added, whereupon it immediately fell to that in the first.

oxypropyl- and α-phenoxyethyl-penicillins as substrates. These results were given in the previous paper (Novick, 1962), and are more accurate than could have been obtained by bio-assay.

Measurement of unknown penicillinase activity. The above procedure was most useful for the study of reaction kinetics with enzyme preparations of known activity. With certain modifications, it could also be used for the estimation of unknown amounts of enzyme. Activities in excess of 1 unit/ml. were ordinarily assayed by the standard procedure of Perret (1954). Lower activities were estimated by the procedure described except that trials were run with different amounts of enzyme until a measurable decolorization rate was obtained. To estimate low activities in complex mixtures with high rates of non-specific iodine absorption, the non-specific absorption had first to be eliminated. Since staphylococcal penicillinase was stable to starch–iodide, this was accomplished by incubating active and heat-inactivated enzyme in separate cuvettes with starch–iodide containing a predetermined excess of iodine. The non-specific absorption was largely complete in 15 min. and the enzyme reaction rate was then measured after the addition of substrate. The iodine concentration was always determined empirically such that sufficient iodine remained for the determination of the enzyme reaction rate. Thus, for gelatin, 2·4 μm-moles of excess iodine were added/mg.; for Hedley Wright broth, 1·2 μmoles/ml.; for CHY medium (Novick, 1962), 0·23 μmole/ml.

In a typical experiment with this technique, 0·5 ml. of Hedley Wright broth to which 0·05 unit of penicillinase activity had been added was incubated for 15 min. at 30° with 2 ml. of starch–iodide (iodine concn. 320 μM). Penicillin G (75 μm-moles in 0·5 ml. of buffer) was then added and the enzyme reaction rate determined. Broth containing an equivalent amount of heat-inactivated enzyme was added to the control cuvette 15 sec. after the active enzyme was added to the experimental cuvette. Substrate was added to the control cuvette 15 sec. later than to the other and there was also a 15 sec. interval between the readings of the two cuvettes. The 15 sec. intervals were necessary because iodine absorption by the broth continued at a measurable rate during the enzyme–substrate reaction. In this experiment, 71 % of the expected activity was observed.

DISCUSSION

The micro-assay described has been useful for the study of penicillinase reaction kinetics and for the detection of low enzyme activities. Its major limitations result from the absorption of iodine by proteins and by other substances present in the complex media in which it may be desired to measure penicillinase activity. Most of the non-specific iodine absorption could be circumvented by incubating the mixture containing enzyme with an excess of starch–iodide before the addition of substrate. However, it must be emphasized that, even after such incubation, the control rate of decolorization was often appreciable, and the timing of all additions and of the readings had to be very carefully controlled.

Along with other proteins, the enzyme itself may be iodinated. However, the penicillinase produced by *S. aureus* 524SC which was used in these experiments was stable to iodine in the presence of starch, and its activity was unchanged. It is therefore unlikely that iodine had any significant effect on the properties of the enzyme measured with this assay. For other penicillinases and for purified staphylococcal penicillinase the effect of iodine would have to be evaluated.

There remains the question of the 40 % discrepancy between measurements of activity by the micro-assay and by the standard method. The evidence presented suggests that the activity of staphylococcal penicillinase is unaffected by starch–iodide and that the enzyme acts at its expected rate under the conditions of the micro-assay. Thus, the 40 % lower activities are probably the result of an error inherent in the determination of *P*, the colorimetric equivalent for penicilloic acid. The difference in conditions between the determination of *P* and the assay itself is that *P* was measured by determining the end point of the reaction between enzyme, starch–iodide and a limiting amount of penicillin, while measurements of activity were taken from linear decolorization rates during the reaction with excess of penicillin. Since the complex and rather sluggish reaction of penicilloic acid and iodine is interposed between the enzyme reaction and the decolorization, the assumption that penicilloic acid has the same iodine equivalent under both sets of circumstances may not be justifiable. If not, the exact standardization of the assay could be achieved by measuring decolorization rates with enzyme of known activity. This, however, would necessitate the determination of enzyme activity by an independent assay method; I have preferred the standardization described, despite the 40 % lower activities, because *P* is easily measured and is reproducible.

SUMMARY

1. A sensitive method for measuring the rate of hydrolysis of penicillin to penicilloic acid by penicillinase is described. It depends upon the reduction of iodine by penicilloic acid but not by

penicillin, and it is carried out by measuring the rate of decolorization of the blue starch–iodide complex when enzyme and substrate react in the presence of starch–iodide.

2. The method was developed for the study of penicillinase reaction kinetics; with it, reaction rates were measured for penicillin concentrations between 1 and 800 μM, and for enzyme activities between 0·001 and 5 μmoles/ml./hr.

3. The micro-assay is 1000 times as sensitive as existing chemical methods, and despite non-specific iodine absorption was useful for the detection and measurement of small amounts of penicillinase in complex mixtures such as broth.

4. Staphylococcal penicillinase was apparently not affected by starch or by iodine under the conditions of the assay, and the method was used for the measurement of the Michaelis constants (K_m) of the enzyme with various substrates.

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The Presence of Non-Protein Nitrogen in Acetic Acid-Soluble Calf-Skin Collagen

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During investigations on the *N*-terminal groups in soluble collagen it was observed that small but significant amounts of non-protein nitrogen were present in the soluble collagen prepared from calf skin. These nitrogenous compounds, now known to be a mixture of amino acids and peptides, donate *N*-terminal groups previously attributed to the protein molecule. The material has been examined by paper chromatography of both the free amino acids and the dinitrophenyl derivatives. It may be separated from the bulk of the protein by acetone precipitation or dialysis at pH 3·5, or by acetone precipitation at pH 11·0 (Steven, Tristram & Tyson, 1961).

Similar compounds have also been found in: (*a*) the collagenous residues remaining after extraction of soluble collagen from calf skin with 0·1 M-citric acid; (*b*) several commercial gelatins [as defined by Ward (1960)].

Non-protein constituents have also been reported in wool (Zahn & Meienhofer, 1955*a*, *b*) and Synge (1953) predicted their occurrence generally in protein systems.

EXPERIMENTAL

Preparation of soluble collagen

Hair was removed from calf skin, which was then minced and suspended in 0·2 M-disodium hydrogen phosphate at room temperature overnight; toluene was added to prevent bacterial contamination. The insoluble residues were separated by centrifuging and further reduced in size by disintegration in a modified hammer-mill or in a commercial-size Waring Blendor. The extraction with phosphate was then repeated five times or until little or no protein was extracted. Collagen was extracted with 0·1 M-acetic acid and precipitated selectively from the pooled acetic acid extracts by the dropwise addition of 30% (w/v) sodium chloride solution with continuous stirring, precipitation of the protein being complete, as a fine fibrous mass, at a final concentration of 7% sodium chloride. After centrifuging, the precipitate was redissolved in 0·1 M-acetic acid, and the protein was again precipitated by the addition of sodium chloride. This procedure was repeated five times to attenuate the non-collagen contaminants. The dry, ash-free protein contained 18% of total nitrogen [determined by the method of Chibnall, Rees & Williams (1943)] and 14% of hydroxyproline [determined by the method of Neuman & Logan as modified by Leach (1960)].